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## Accepted Manuscript

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**The progressive onset of cholinergic and adrenergic control of heart rate during development in the green iguana, *Iguana iguana*.**

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**List of abbreviations:**

Ach - acetylcholine

Adr – adrenergic

Atr – atropine

Chol – cholinergic

 $f_H$  – heart rate

Hyp – hypoxia

Hyperc – hypercapnia

Prop – propranolol

Recov – recovery

**Abstract**

The autonomic control of heart rate was studied throughout development in embryos of the green iguana, *Iguana iguana* by applying receptor agonists and antagonists of the parasympathetic and sympathetic systems. In early embryos (< 10 days incubation) the chronotropic response to drugs topically applied to the heart was visually recorded while in later embryos drugs were injected into the amniotic fluid, with heart rate recorded remotely using an infrared monitor. Acetylcholine (Ach) slowed or stopped the heart and atropine antagonized the response to Ach indicating the presence of muscarinic cholinergic receptors on the heart of early embryos. However, atropine injections had no impact on heart rate until immediately before hatching, when it increased heart rate by 15%. This cholinergic tonus increased to 34% in hatchlings and dropped to 24% in adult iguanas. Although epinephrine was without effect, injection of propranolol slowed the heart throughout development, indicating the presence of  $\beta$ -adrenergic receptors on the heart of early embryos, possibly stimulated by high levels of circulating catecholamines. The calculated excitatory tonus varied between 33-68% until immediately before hatching when it fell to 25-29%, a level retained in hatchlings and adults. Hypoxia caused a bradycardia in early embryos that was unaffected by injection of atropine indicating that hypoxia has a direct effect upon the heart. In later embryos and hatchlings hypoxia caused a tachycardia that was unaffected by injection of atropine. Subsequent injection of propranolol reduced heart rate both uncovering a hypoxic bradycardia in late embryos and abolishing tachycardia in hatchlings. Hypercapnia was without effect on heart rate in late stage embryos and in hatchlings.

**Key words:** Squamata, iguana, embryos, development, cardiovascular, hypoxia, hypercapnia, hatchlings

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## 1. Introduction

The morphological and physiological changes accompanying embryonic development in reptiles are relatively poorly understood. This is in contrast to our knowledge of these processes in other oviparous vertebrates such as amphibians (Burggren, 1995; Burggren and Doyle, 1986) and birds (Crossley and Altimiras, 2000; Crossley et al., 2002; Crossley et al., 2003a). The paucity of data for reptiles invites attention as they represent a key evolutionary link between the ancestral amphibians, birds and mammals. By far the most tractable cardiovascular parameter that can be measured in embryonic reptiles is heart rate ( $f_H$ ), using non- or minimally invasive methods. These methods can be used to monitor the onset of central nervous control of heart rate via the autonomic nervous system, using appropriate pharmacological agents.

The ontogenetic development of  $f_H$  regulation in reptiles has been investigated primarily in American alligators, *Alligator mississippiensis* and common snapping turtles, *Chelydra serpentina*. Two main characteristics have emerged. Embryonic alligators maintain a constant and pronounced  $\beta$ -adrenergic tone throughout the final 40% of incubation that is attributable to circulating catecholamines and not mediated by an adrenergic innervation. They also lack cholinergic tone mediated by cholinceptors until the time of hatching (Eme et al., 2011). Alligator embryos also lack central nervous control of  $f_H$  in response to hypoxia and exhibit a limited hypertensive baroreflex response (Crossley et al., 2003b; Crossley and Altimiras, 2005). We have recently obtained similar findings in the Paraguayan caiman (*Caiman yacare*) (Crossley et al., unpublished), possibly indicating this is a common feature of development in crocodilians. Like alligators, embryonic turtles possess a marked  $\beta$ -adrenergic tone on heart rate at 70% of incubation that was not attributable to sympathetic nervous outflow (Eme et al., 2013). Heart rate falls slightly before and markedly after hatching indicating the establishment of a vagal tone on the heart coincident with the onset of lung breathing (Birchard and Reiber, 1996). A number of turtles and tortoises lack cholinergic tone on  $f_H$  until the time of hatching (Crossley and Burggren, 2009; Taylor et al., 2014), although cholinergic tonus was verified during the final 30% of embryonic incubation in snapping turtles (Alvine et al., 2013). One preliminary study of the African brown housesnake *Boaedon fuliginosus* (Crossley and Burggren, 2009) suggested that cholinergic tone may be present prior to hatching. However, an extensive assessment of the onset of cholinergic tone on the heart has yet to be conducted in squamates.

The present study focused on measurement of  $f_H$  during embryonic development in the green iguana, *Iguana iguana*. The injection of appropriate agonists and antagonists, as well as exposure to changed levels of respiratory gases was used to reveal the onset and degree of autonomic control of the cardiovascular system in embryos and hatchlings. This is the first study of this aspect of development to provide data from a member of the superorder Lepidosauria that includes the extant lizards and snakes (the squamates) and is further distinguished by having extended the study to early embryos (< 10% incubation).

## 2. Materials and Methods

### 2.1 Experimental animals

The eggs and adults for this study were supplied from the breeding program operated by Prof Abe, his staff and students at the Jacarezário, Departamento de Zoologia, UNESP, Rio Claro, SP. Adult iguanas (*Iguana iguana*) were maintained in a large, outdoor vivarium with water *ad libitum* and fed daily with vegetables and fruits. Each year of the study clutches of eggs ranging in numbers from 15-50 eggs, were collected during the months of September and October. Eggs were weighed, placed in trays (38 x 28.5 x 6.5 cm) containing vermiculite and placed at a constant temperature of 30°C  $\pm$  0.5°C in incubators (Eletrolab, EL101/3, SP, Brazil). All eggs were examined daily for signs of mortality and the vermiculite sprayed with dechlorinated tap water to maintain humidity high. Eggs were selected for physiological study at 5-8 days, 18-20 days, 29-31 days, 43 days, 51-53 days and finally 67-71 days, listed as groups 1 to 6. For the present study we have identified our 6 groups according to the stages provided by Sanger et al. (2008) (Table 1). Group 6 animals had developed to a point immediately before hatching later confirmed by successful hatching of a subset of eggs. At the conclusion of experiments, embryos at all phases of development were killed by extended exposure to greatly increased levels of CO<sub>2</sub> (>20%) and were then preserved for separate morphological study. Hatchlings were held for experimentation for up to 90 days after hatching.

### 2.2 Experimental protocol

#### 2.2.1 Studies on iguana embryos

A total of 107 embryos were used during this study. All experimental procedures were approved by the Local Ethics Committee on Animal Care at the São Paulo State University (CEUA-UNESP no. 6597). Prior to experimentation, eggs were weighed and heart rate was recorded to determine baseline values. For the earliest embryos (group 1) each egg was first “candled” over a bright, cold, fiber-optic light source to detect the position of the embryo. An approximately  $1\text{cm}^2$  window was then cut through the eggshell to view the heart, which at this early stage of development was not yet enclosed within the body wall. Heart rate was counted visually by means of a dissecting microscope (Stemi 2000 C, Zeiss, Germany), at room temperature ( $28\pm 1^\circ\text{C}$ ). In these experiments drugs (up to 0.2 ml) were applied topically to the heart surface via a fine 27-gauge hypodermic needle. In later stages the heart had increased in size and become enclosed within the body wall of the growing embryo so that it was no longer visible. Attempts to measure an ECG or cannulate CAM (chorioallantoic membrane) vessels to measure blood pressure proved ineffective. Consequently, the heart’s activity was recorded non-invasively using a digital egg monitoring system (Buddy System, Avitronics, Truro, UK), that recorded movements of the heart from outside of the egg, as described for birds by Tazawa et al (1994) and for reptiles by Du et al (2009). The Buddy system was customized by the supplier with an additional analog output at our request. This output was connected to a Powerlab system (ADInstruments, Sydney, Australia) with  $f_H$  recorded continuously, using data acquisition software (LabChart 7, ADInstruments). Each egg was placed in the monitoring device and surrounded by a ring of water saturated gauze. The device was then placed inside an incubator (Caltech EIP-010, PE, Brazil) set at  $30\pm 0.5^\circ\text{C}$  for at least 1 hour prior to recording. Baseline  $f_H$  was then recorded prior to each drug injection. In these experiments a portion of the egg shell was removed in order to assist selective injection of drugs close to the embryo and the lid of the monitor was opened during injection.

*Drug injections:* In early embryos (group 1) topical injection onto the heart was used for application of acetylcholine (Ach), the agonist for cholinergic receptors, and adrenaline (Adr), the agonist for adrenergic receptors, followed by the appropriate antagonists, atropine (Atr) or propranolol (Prop). All were applied drop-wise to the heart as solutions in physiological saline at a range of concentrations up to  $10^{-3}$  molar. In later embryos (after 18 days of incubation) drugs were applied initially by opening



a window in the shell and injecting the drugs in proximity to the developing embryo or through its body wall. Later, this relatively more invasive technique was replaced by injection through the eggshell. The egg was first “candled” as described above, to detect the position of the embryo and its yolk sac and an injection site close to the embryo that avoided injection into the yolk sac was marked on the outside of the egg. The drugs were then injected via this site into the amniotic fluid from whence they were absorbed into the embryonic circulation. After the baseline  $f_H$  was established a control injection of saline was given and responses recorded. This was followed by an injection of, 0.2 ml of the muscarinic cholinergic antagonist atropine ( $10^{-3}$  mol), and then the  $\beta$ -adrenergic antagonist propranolol ( $10^{-3}$  mol) (Sigma Aldrich) in close proximity to embryos at developmental stages from group 2 to 6. To ensure that the full effects of the drugs were recorded measurements were taken for up to one hour after the initial injection. The efficacy of each antagonist was verified by subsequent injection of the appropriate agonist or a second dose of the antagonist. Injection of drugs necessitated the opening of the lid of the egg monitoring system, causing brief changes in the local environment surrounding the egg.

*Heart rate response to changes in respiratory gases:* In order to uncover cardiac responses to changes in respiratory gas concentrations, early (group 2) and late stage (group 5-6) embryos were exposed to 5% O<sub>2</sub> (hypoxia) or 5% CO<sub>2</sub> (hypercapnia). Exposure to different gas mixtures was achieved by enclosing each egg monitoring system containing an egg in a sealed plastic bag (16 x 26.5 cm) supplied with gas mixtures produced by calibrated flowmeters (Cole Parmer, Vernon Hills, USA). The exit tubes were led to gas analyzing equipment (PA-10 and CA-10, Sable Systems, Las Vegas, USA) in order to ensure that the eggs were exposed to the correct experimental gas mixtures. Each gas mixture was applied to the eggs for 1 hr followed by a period of 30-60 min of recovery in air. During this series the lid of the monitor remained closed for the duration of each experiment. In a subset of studies egg temperature was recorded by inserting a thermocouple (T-type implantable thermocouple, ADInstruments) through the eggshell and membranes, to lie up to 5 mm into the amniotic fluid, clear of the embryo and yolk sac. Output from the egg monitoring systems and the thermocouple signals were connected to an interface (PowerLab plus Animal Bio Amp, ADInstruments, Sydney, Australia) and recorded with data acquisition software (LabChart 7, ADInstruments, Sydney, Australia).

These experiments revealed that the infrared detection of  $f_H$  by the egg monitoring system causes a progressive increase in egg temperature. This warming effect was particularly intense in this second series of experiments, performed with the lid closed for prolonged periods, when egg temperature increased from a 28°C to 32°C over a period of 1 hour. However, if the lid was held open the temperature of the egg remained below 30°C for 2 hours. These changes in temperature of up to 2°C either side of the incubation temperature of 30°C affected  $f_H$  (Sartori et al., 2015b). Their possible confounding effects on our study were mitigated by comparing all changes in  $f_H$  accompanying drug injection or changes in respiratory gas concentrations with baseline rates recorded immediately prior to and on recovery from treatment.

### 2.2.2 Studies on hatchlings

For measurement of  $f_H$  following drug injection or exposure to different gas mixtures iguana hatchlings (2-3 months) were instrumented with two lengths of Teflon insulated silver wire (A-M Systems, WA, USA), inserted subcutaneously either side of the mid-thoracic wall. The hatchlings were enclosed in 50 ml Falcon tubes (Cralplast, SP, Brazil) opened at each end to allow the anterior portion of the head and the tail to extend outside of the tube. The tubes containing the hatchlings were placed in an incubator set at 30°C (Caltech EIP-010, PE, Brazil). The wire leads were externalized via a slot cut through the wall of the Falcon tube and connected to a preamplifier (Animal Bio Amp, ADInstruments). Raw data were recorded and selectively filtered, using data acquisition software (LabChart 7, ADInstruments) to separate the ECG and ventilatory signals. Data were collected overnight to determine the baseline  $f_H$ , then autonomic regulation of  $f_H$  was determined by injection of 0.25 ml of saline or the respective antagonists atropine ( $10^{-3}$  mol) or propranolol ( $10^{-3}$  mol) into the peritoneal cavity. In order to check complete blockade second doses of each drug were injected. In a separate series of experiments  $f_H$  responses to 10 min exposure to 10% O<sub>2</sub> or 5% CO<sub>2</sub> followed by a period of 30-60 min of recovery in air were accomplished as outlined for the embryonic studies.

### 2.2.3 Studies on juvenile and adult iguanas

Juvenile and adult iguanas (N= 7, mass= 902.6±60.3g) were first lightly anaesthetized in an atmosphere of CO<sub>2</sub> (see Wang et al, 1993; Taylor et al., 2009) then held under anesthesia on a respiratory pump (CWE, SAR-830/P Ventilator, USA) supplied

initially with 5% isoflurane. This dose was reduced to 2% once anesthesia was established. The femoral vein was occlusively cannulated for injection of drugs as previously described (Busk et al., 2000). ECG electrodes were placed under the skin on opposing lateral sides of the thorax rostral or caudal to the heart and a reference electrode inserted dorsally, 1 cm lateral of the vertebral column. All leads were anchored to the back of the animal with a suture. The area around all operative lesions and suture points was injected with a local anesthetic (Lidocaine, Eurofarma, SP, Brazil). The animal was then placed in an incubator set at 30°C (Caltech EIP-010, PE, Brazil) for recovery and the catheter and ECG leads were led through a port in the wall. Animals were then left overnight in the incubator to recover from the effects of anesthesia and handling. The leads were connected to an ECG amplifier (Animal Bio Amp, ADInstruments) and raw signals were collected and processed as described for the hatchlings. Following measurements of baseline  $f_H$  1.0 ml of atropine ( $10^{-2}$  mol) or propranolol ( $10^{-2}$  mol) was injected via the femoral vein and resultant changes in  $f_H$  recorded for up to one hour post-injection, for calculation of autonomic tones.

### 2.3 Statistical Analysis

The study of autonomic tones used a total of 74 eggs, 6 hatchlings and 7 adults. The responses to changes in respiratory gases used a total of 33 eggs and 12 hatchlings. All statistical tests were conducted with the software package SigmaPlot v. 11. The effects of drug injections within each group, the comparison of adrenergic and cholinergic tonus between different groups and  $f_H$  changes in response to hypoxia and hypercapnia with or without blockade with drugs were all tested with one-way ANOVA. Student-Newman-Keuls (SNK) test was used for *post-hoc* comparisons, and each treatment was compared with the previous value. When the data failed a test of normality an ANOVA on Ranks and Dunn's test was used. Significance was attributed to any changes at the 5% level of confidence ( $P < 0.05$ ).

## 3. Results

### 3.1 The embryos

A profile of the embryonic groups used in the study is detailed in Table 1. Egg mass from the first to the last group increased two-fold (from  $15.4 \pm 0.5$  g to  $31.0 \pm 1.3$  g) and embryo wet mass increased 60-fold (from  $0.16 \pm 0.01$  g to  $9.76 \pm 0.13$  g). The embryos

were staged according to the staging table for *Anolis sagrei*, a lizard species closely related to the green iguana (Sanger et al., 2008).

*Baseline embryonic heart rate:* Baseline  $f_H$  in group 1 embryos were measured by direct visual observation at room temperature (28°C). They were significantly lower than baseline  $f_H$  in the succeeding groups 2-6 that were recorded using the egg monitoring system held inside the incubator at 30°C. The mean rates were statistically similar within these latter groups (Table 2).

#### *Effects of drug injection on embryo heart rate*

*Cholinergic blockade:* Early embryos showed a clear, dose-dependent reduction in  $f_H$  to topic application of acetylcholine (Ach). This culminated in cardiac arrest at  $10^{-2}$  molar (Figure 1). This effect was reversed by application of atropine, with  $f_H$  progressively recovering to the rate prior to injection of acetylcholine (Figures 1 and 2). Following atropine injection subsequent injection of acetylcholine had no effect (data not shown). This cholinergic inhibition was apparent in all embryos. However, injection of atropine alone into the eggs had no effect on all embryos from groups 1 to 5 (Table 2; Figures 2 and 3). Mean values of atropinized  $f_H$  are shown in Table 2 and also illustrated for groups 2-5 in Figure 3, as this shows the effect of prior injection of saline as a control for the injection of atropine. In contrast to groups 2-5, group 6 (67-71 days) embryos showed a significant increase of 17% in  $f_H$  following injection of atropine (Figure 4).

*Adrenergic blockade:* Topic application of adrenaline to early-stage embryos and injection into the amniotic fluid of later embryos had no effect on  $f_H$  (Fig. 2) but injection of Propranolol markedly reduced  $f_H$  at all stages of embryological development (Table 2; Figures 2, 3 and 4). Based on  $f_H$  values following atropine injection, the decrease in  $f_H$  induced by propranolol was approximately 45% in groups 1 to 5 and 32% in group 6.

#### *3.2 Effects of drug injection on heart rate in hatchling iguanas:*

Recordings of  $f_H$  in hatchlings revealed mean resting values of  $43 \pm 1$  bpm interrupted by brief bouts of tachycardia associated with spontaneous movements. Atropine injections increased  $f_H$  to  $60 \pm 2$  bpm representing an increase of 41% (Table 2). Propranolol injections reduced  $f_H$  to a mean of  $43 \pm 5$  bpm, representing a decrease of

28% from the atropinized value. The effectiveness of these treatments was verified by repeated injections of each antagonist into the peritoneal cavity.

### *3.3 Effects of drug injection on heart rate in adult iguanas:*

Mean  $f_H$  after 24h of recovery from surgery was  $28 \pm 1$  bpm (Table 2). Intravenous injection of atropine (1ml of  $10^{-2}$  molar) caused an increase of 38% in  $f_H$  (mean =  $37 \pm 2$  bpm). Subsequent injection of propranolol at a similar dose caused a decreased  $f_H$  from the elevated rate following atropine injection to a mean of  $28 \pm 2$  bpm, corresponding to a 24% decrease. Effectiveness of injection of both antagonists was verified by the intravenous injection of the appropriate agonist.

### *3.4 Calculation of autonomic tones:*

Table 2 summarizes  $f_H$  before and after injection of atropine and propranolol in the 6 embryonic groups, hatchlings and adults. The adrenergic tonus on the hearts of all groups, plus the cholinergic tonus on the hearts of group 6 embryos, hatchlings and adult iguanas were calculated from the combined means of the mean cardiac intervals collected for individuals in each group (see Altimiras et al, 1997). Embryos in groups 1-6 had a clear adrenergic tone on the heart that varied between 33 and 68%, with the highest value found in group 3 that differed statistically from group 6 embryos, hatchlings and adults. Hatchlings had an adrenergic tone of 25% and adults of 29%. A cholinergic tone of 15% was first apparent in group 6 embryos, increasing to 34% in hatchlings and stabilising to 24% in inactive adults (Figure 5).

### *3.5 Effects of changes in the levels of respiratory gases*

Exposure to 5% oxygen in early stage embryos (group 2) reduced mean  $f_H$  from  $74 \pm 4$  to  $52 \pm 3$  bpm, representing a 30% change that recovered in subsequent normoxia (data not shown). Initial  $f_H$  was unaffected by prior injection of atropine and hypoxia still elicited a decrease of 21% (Figure 6). In contrast, exposure of late embryos (groups 5-6) to hypoxia caused a small but significant initial tachycardia, increasing  $f_H$  from  $115 \pm 1$  to  $125 \pm 1$  bpm, representing a 8% change that was unaffected by injection of atropine (Figure 7A). Injection of propranolol caused an overall reduction in  $f_H$  (from  $114 \pm 3$  to  $71 \pm 3$  bpm) and abolished the initial tachycardia on hypoxic exposure, revealing a bradycardia of 30%, when  $f_H$  decreased to a value of  $50 \pm 3$  bpm (Figure 7B). Hatchlings showed a hypoxic tachycardia of 25% (from  $44 \pm 1$  to  $55 \pm 4$  bpm) that

was unaffected by injection of atropine but abolished by propranolol (Figure 8). Exposure to 5% CO<sub>2</sub> of late (groups 5-6) embryos (data not shown) and hatchlings (Figure 8) was without effect on  $f_H$  despite a marked hyperventilation in the latter group (data not shown).

## 4. Discussion

### 4.1 Measurement of baseline heart rates

This is the first study of the ontogeny of cholinergic and adrenergic control of heart rate ( $f_H$ ) throughout development in a squamate reptile, the green iguana, *Iguana iguana*. It differs from other studies as the responses to drug injection were measured in a wide range of developmental stages from very early embryos (< 10% incubation time) to immediately prior to hatching, as well as in hatchlings and adult lizards. Although we consider the data to be sufficiently reliable to enable us to describe the progressive development of autonomic control in this species, the data were complicated by varying temperature during experiments. The data on group 1 embryos was collected at room temperature (28°C), while all subsequent embryonic groups were studied by enclosure of each egg in a monitoring device (the Buddy system, Avitronics, UK) that used low levels of infrared radiation to measure heart beats. Complete enclosure in the device resulted in progressive warming of the eggs from room temperature to a maximum of 32°C over about 1 hour (Sartori et al., 2015b). Accordingly, initial  $f_H$  varied between experiments (Table 2; Figures 6 and 7). However, the potential for obscuring the effects of drug injection were avoided by a control injection of saline immediately prior to each drug injection, while exposure to changes in respiratory gases were compared to individual initial rates prior to exposure and expressed as % age changes. Reptilian eggs are routinely subjected to temperature variations in their natural environment on a daily and seasonal basis (Ackerman and Lott, 2004).

### 4.2 Injection of drugs to reveal onset of autonomic control

The different routes of administration of drugs (i.e. topical application onto the hearts of very early embryos, injection in the amniotic fluid in later embryos, injection into the peritoneal cavity in hatchlings and injection through a cannula in the femoral vein

in adults) could have resulted in different degrees of dilution of the drug. However, the doses were relatively high and subsequent injection of agonists or a second dose of the antagonist revealed that each antagonist had provided complete blockade of the receptors for the duration of the experiment.

Topic application of the cholinergic agonist acetylcholine markedly affected  $f_H$  in group 1 embryos, within the first 10% of incubation time, causing cardiac arrest at  $10^{-2}$  molar concentration. This response was abolished by prior or subsequent injection of the antagonist atropine. These data show that the embryonic iguana heart possesses muscarinic cholinergic receptors at the earliest stage in development, post-oviposition. Similar results were obtained from embryos at all stages of development, though the sensitivity to acetylcholine varied between clutches. Despite this clear demonstration of the existence of muscarinic cholinergic receptors on the heart of early and mid-stage (1-4) iguana embryos, injection of atropine alone was without effect on  $f_H$ , revealing an absence of an inhibitory cholinergic tone on the heart. Only in group 6 embryos that had undergone more than 90% of development was there evidence of a cholinergic tone that increased in hatchlings and adults (Figure 5). These data were previously unavailable from squamate embryos, though a similar finding has been reported as unpublished data from the African brown house snake, *Boaedon fuliginosus* (Crossley and Burggren, 2009). The lack of a cholinergic tone on the heart, despite the recorded presence of receptors, implies lack of establishment of appropriate connections within the CNS. Alternatively, the vagal efferent supply to the heart may not yet have established functional connections with the heart or cardiac ganglion. It is also possible that the nerve fibers in early embryos lack the myelination required to confer a conduction velocity sufficient to affect beat-to-beat modulation of the heart (Foster et al., 1982; Crossley and Altamiras, 2000; Taylor et al., 2014). As cholinergic tone is often not detectable in late stage vertebrate embryos until close to or immediately after hatching or birth in a range of species (Taylor et al., 2014), we hypothesize that this may relate to the onset of respiratory rhythmicity or physical respiratory movements. In the red footed tortoise it appears during embryonic development (Crossley et al., 2013), as described here for the iguana while in the chicken cholinergic control appears post-hatching (Crossley and Altamiras, 2000). A previous hypothesis suggested that these differences in timing of the appearance of cholinergic control of  $f_H$  between species relates to the varying provision of maternal

care to the hatchlings of birds and reptiles. Chicks receiving a high level of maternal care immediately post-hatching show a delay in the full establishment of central control of  $f_H$  whereas reptiles lacking this care may require effective central control of  $f_H$  prior to hatching to ensure survival (Crossley and Altimiras, 2000).

Although the embryonic iguana heart was insensitive to injection of adrenaline  $\beta$ -adrenergic blockade by injection of propranolol markedly reduced heart rate at all stages of embryonic development (Table 2, Figs. 2, 3 and 4). This demonstrates that the heart possesses  $\beta$ -adrenergic receptors from an early stage. There is evidence that many vertebrate embryos have both muscarinic cholinergic and  $\beta$ -adrenergic receptors on the heart at an early stage of development (Taylor et al., 2014). Cardiac muscarinic and adrenergic receptors were found to be present in embryonic chickens during the first quarter of incubation (Berry, 1950) and the enzymes responsible for the production or breakdown of the neurotransmitters acetylcholine and noradrenaline were also present during early development in chickens (Zacks, 1954; Ignarro and Shideman, 1968). The existence of an adrenergic tone on the embryonic heart may indicate high levels of circulating catecholamines as described for embryonic chickens (Crossley and Altimiras, 2000) and alligators (Eme et al., 2011). Lack of response to injection of adrenaline in iguana embryos may indicate these high levels of circulating catecholamines saturated the  $\beta$ -adrenergic receptors on the heart. However, it is also possible that the injected adrenaline was oxidized during its passage from the amniotic fluid to the receptors on the heart.

#### 4.3 Autonomic Tonus

Embryos prior to 90% of incubation lacked cholinergic tone but had a clear adrenergic tone on the heart that varied between 38 and 68%. Just prior to hatching this was 33% while a cholinergic tone of 15% then became apparent (Fig. 5). Hatchlings had an adrenergic tone of 25% and a cholinergic tone of 34% while in adults the adrenergic tone was 29% and cholinergic tone was 24% (Figure 5).

Thus, the onset of a cholinergic tonus, implying vagal control of the heart, in late stage iguana embryos was accompanied by the establishment of a level of adrenergic tone that closely resembled the level in hatchling and adult iguanas. This may indicate a switch from control exerted by high levels of circulating catecholamines to nervous control exerted by the CNS via the sympathetic innervation of the heart. While these



data provide a glimpse of the ontogeny of cardiac regulation in a single species of squamate reptile the limited information available constrains speculation regarding the commonalities within this order but provides a fertile ground for future studies.

Regulation of  $f_H$  in embryonic reptiles has been investigated primarily in American alligators and common snapping turtles. Two characteristics of regulation seem to emerge in the species investigated to date (see Taylor et al., 2014). In the American alligator tonic cholinergic receptor mediated regulation is absent until just prior to or after hatching (Eme et al., 2011). Alligator embryos also lack nervous control of the  $f_H$  response to hypoxia and exhibit a limited hypertensive baroreflex response (Crossley et al., 2003b; Crossley and Altimiras, 2005). They do, however, maintain a pronounced  $\beta$ -adrenergic cardiac tone of constant intensity, elevating  $f_H$  throughout at least the final 40% of incubation (Crossley et al, unpublished; Eme et al., 2011) but this is not mediated by sympathetic nervous system output, relying instead on hormonal regulation by circulating catecholamines (Eme et al., 2011). Unlike alligators, common snapping turtles (*Chelydra serpentina*) possess a clear cholinergic tone, depressing  $f_H$  over the final 30% of embryonic incubation. However, this is not a general Testudine characteristic as embryonic desert tortoise (*Gopherus agassizi*), Red-footed tortoise (*Chelonoidis carbonaria*), Red-eared slider turtles (*Trachemys scripta*) and D'Orbigny's slider (*Trachemys dorbigni*) lack cholinergic tone on embryonic  $f_H$  (Crossley and Burggren 2009; Crossley et al., 2013; Sartori, unpublished). However, the presence of tonic cholinergic depression of  $f_H$  has been identified in *C. carbonaria* just prior to hatching (the final 5% of incubation) and this may also be the case for the other 3 species studied (Crossley et al., 2013). Like the alligators, embryonic snapping turtles also possess a marked  $\beta$ -adrenergic tone on  $f_H$  that does not appear to originate from sympathetic nervous outflow (Eme et al., 2013). Similar  $\beta$ -adrenoceptor stimulation on  $f_H$  has been reported for *G. agassizi* (Crossley and Burggren, 2009), *C. carbonaria* (Crossley et al., 2013), *T. scripta* (Sartori, unpublished) and *T. dorbigni* (Sartori, unpublished). Thus while the onset of cholinergic receptor tone seems species-specific, the presence of  $\beta$ -adrenergic tone on  $f_H$  is a shared feature of the Testudine species studied. The possibility of control exerted by nonadrenergic and noncholinergic (NANC) factors has been considered and a role for histamine via H1 and H2 receptors was identified in the red-footed tortoise, *Chelonoidis carbonaria* (Crossley et al., 2013).

#### 4.4 Responses to changes in respiratory gases

Exposure to hypoxia caused a significant bradycardia in early iguana embryos. However, this response was intact following injection of atropine suggesting that it was the result of a direct effect upon the heart rather than a reflex arising from stimulation of chemoreceptors. Hypoxia also caused a marked bradycardia in embryos of the chicken. Early in development (day 12 of incubation) this hypoxic bradycardia appeared to arise from the direct effect of low oxygen on the cardiac muscle. Until day 18 of incubation there was no evidence for the change in  $f_H$  in hypoxia being a reflexive response involving the CNS. However, immediately pre-hatching, at day 21, injection of atropine limited the hypoxic bradycardia indicating that there was a component of the cardiac chronotropic response that was generated from the CNS via the parasympathetic nervous supply to the heart (Crossley et al., 2003c). This suggests that, contrary to the conclusion arrived at above, the chick does possess some cholinergic control of the heart established immediately before it hatches that is only revealed by hypoxic exposure. This was accompanied by a late onset of neural adrenergic control as is suggested by our data on iguana embryos. However, in late (group 5-6) iguana embryos hypoxia did not cause a bradycardia, indeed the response was a small but significant tachycardia, although a bradycardia was revealed after adrenergic blockade. Hatchlings also showed a tachycardia during hypoxia that was not affected by injection of atropine but was reduced by injection of propranolol. In neither stage was  $f_H$  affected by hypercapnia although hatchlings showed periods of marked hyperventilation. These data raise interesting questions regarding the onset of the secondary response to hypoxia in an air-breathing species. This comprises the masking of a reflex bradycardia during hypoxia by a secondary tachycardia in response to increased lung ventilation (Spyer, 1982; Daly, 1986). This explains the tachycardia in hatchlings, but not that displayed by group 6 embryos unless they had commenced initial lung ventilation. The absence of a cardiac response to hypercapnia in hatchlings, despite evident hyperventilation, also questions the development of the full spectrum of air-breathing responses in this species. These remain open questions.

## 5. Conclusion

A representation of the time course of recorded changes in the factors affecting  $f_H$  in embryonic iguanas is provided in Figure 9. These factors are plotted against %age of total incubation time to enable comparison with similar diagrams provided for chicken and emu (Burggren and Crossley, 2002). The figure reveals that an excitatory adrenergic tone is present on the heart throughout development in all three species. In addition, they all show evidence for muscarinic cholinceptors on the heart from an early stage in development. However, the onset of an inhibitory tone varies between species with the iguana acquiring it prior to hatching while the normoxic chicken delays onset until post-hatching. Observation of the timelines for the onset of autonomic control of the heart in vertebrate embryos provided by Taylor et al. (2014) reveals that all show a persistent presence of an excitatory adrenergic tonus throughout development. However, half the species studied, including birds, reptiles and a fish, showed delayed onset of an inhibitory, cholinergic tonus, to just before or even in some species beyond hatching. Human babies show the onset of respiratory sinus arrhythmia, indicative of cholinergic vagal control of  $f_H$  at 85% of gestation (Taylor et al., 2010) supporting the conclusion that late onset of this control immediately anticipates the need for control of cardiorespiratory interactions in the newly independent individual. A more thorough examination of the different developmental strategies must await investigation of the onset of chemoreceptive and baroreceptive control of the cardiovascular system in a range of species, including the iguana. This will require cannulation of CAM vessels, which is proving very difficult and is the reason why this investigation came to rely on remote monitoring of  $f_H$ .

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**Table 1** Profile of the iguana embryonic groups (1-6) used in this study differentiated by incubation time, expressed in days from oviposition, % age incubation time and as stage by comparison with the sequence described by Sanger et al. (2008), plus mean egg mass $\pm$  SEM. Number of observations is given in Table 2.

Group	Incubation time (days)	% of Incubation	Stage	Egg mass (g)
1	5 to 7	5-10 %	4	15.4 $\pm$ 0.5
2	18 to 21	25-30%	10	23.5 $\pm$ 0.5
3	29 to 31	35-40%	13	27.8 $\pm$ 1
4	43	60%	14	30.1 $\pm$ 0.8
5	51 to 53	65-70%	17	35.1 $\pm$ 1.1
6	67 to 71	95-100%	18	31 $\pm$ 1.3

**Table 2** Effects of autonomic blockade on  $f_H$  in embryonic groups 1-6 (see Table 1), hatchlings (Hatchl) and adult iguanas. Values expressed as mean heart rates  $\pm$  SEM, respective number of observations (N), and mean body mass. (\*) indicates significant differences within  $f_H$  of each row, and (\*\*) indicates significant differences within  $f_H$  of the embryonic groups.

Group	N	Body Mass (g)	$f_H$ Settled (bpm)	$f_H$ Post Atr (bpm)	$f_H$ Post Double Block (bpm)
1	26	0.16 $\pm$ 0.01	47.9 $\pm$ 1.5 (**)	43.2 $\pm$ 1.5	24.0 $\pm$ 4.4 (*)
2	10	0.61 $\pm$ 0.03	80.5 $\pm$ 2.2	88.3 $\pm$ 4.2	47.7 $\pm$ 8.7 (*)
3	7	1.26 $\pm$ 0.09	73.3 $\pm$ 2.8	70.8 $\pm$ 8.1	43.4 $\pm$ 2.8 (*)
4	8	2.57 $\pm$ 0.06	79.1 $\pm$ 4.8	80.6 $\pm$ 6.5	44.5 $\pm$ 11.4 (*)
5	7	4.57 $\pm$ 0.50	65.1 $\pm$ 4.0	61.5 $\pm$ 9.5	34.7 $\pm$ 8 (*)
6	16	9.76 $\pm$ 0.13	79.5 $\pm$ 5.2	93.3 $\pm$ 4.9 (*)	63.4 $\pm$ 4.3 (*)
Hatchl	6	13.4 $\pm$ 0.2	42.5 $\pm$ 1.3	60.8 $\pm$ 1.8 (*)	43.2 $\pm$ 4.6 (*)
Adult	7	902.6 $\pm$ 60.3	28.4 $\pm$ 1.3	37.2 $\pm$ 2.2 (*)	27.6 $\pm$ 1.7 (*)

## Figure captions

**Figure 1.** Recording of heart beats from a 19 day iguana embryo (group 2; egg mass 22.56 g) with the egg monitoring system (Buddy System; upper trace) and an instantaneous calculation of heart rate from the data acquisition interface (PowerLab; lower trace). Injection of acetylcholine (Ach) reduced the apparent force of recorded heart beats then caused cardiac arrest. Subsequent injection of atropine (Atr) resulted in a progressive recovery in the force and rate of heart beats back to the value measured before injection of Ach. The recording was interrupted for 3 min then 5 min as indicated respectively by the arrows.

**Figure 2.** Mean values of heart rate ( $f_H$ ) for early iguana embryos (group 1; 5-8 days post oviposition; N=26). The first column represents the initial  $f_H$ , 10 minutes after opening the egg (Init); then following topic application of saline (Sal), acetylcholine (Ach); atropine (Atr); adrenaline (Epi) and propranolol (Prop). Ach caused a significant decrease in  $f_H$  that was abolished by the subsequent Atr injection. Although Epi was without effect, Prop caused a significant reduction in  $f_H$ .

**Figure 3.** Mean values ( $\pm$  SEM) of heart rate for iguana embryos of groups 2 (N=10), 3 (N=7), 4 (N=8) and 5 (N=7). The first column (Init) represents the stabilized  $f_H$  achieved one hour after placement in the egg monitoring system and following columns represents  $f_H$  values after injection of saline, atropine then propranolol (labelling as for figure 2). Propranolol caused a significant reduction in  $f_H$  in all groups, but atropine was without effect.

**Figure 4.** Mean values ( $\pm$  SEM) of heart rate ( $f_H$ ) for late embryos of iguana (group 6; 67 – 71days after oviposition; N=16). Saline (Sal) injection was compared to initial (Init) values and there was no significant change in  $f_H$ . Atropine (Atr) injection was compared to a post-saline injection and a significant increase in  $f_H$  was detected. Propranolol (Prop) was compared to a post-atropine value and a significant reduction in  $f_H$  was detected.

**Figure 5.** Mean values ( $\pm$  SEM) of adrenergic and cholinergic tonus on iguana embryos (groups 1 to 6), hatchlings (H) and adults (A). There is an excitatory



adrenergic tone on the heart of all embryos groups that is significantly higher in group 3 (asterisk symbol,  $P = 0.001$ ) when compared to group 6, hatchlings and adult animals. An inhibitory cholinergic tone was first apparent in group 6 embryos and this increased significantly in hatchlings but is not different in adult animals, as denoted by the different letters ( $P=0.003$ ).

**Figure 6.** Mean values ( $\pm$  SEM) of heart rates ( $f_H$ ) of iguana embryos from group 2 (18-20 days;  $N=8$ ) exposed to hypoxia following injection of atropine.  $f_H$  was significantly reduced by hypoxia after atropine injection ( $p < 0.05$ ).

**Figure 7A.** Effect of exposure to 5% oxygen (Hyp 5%) on heart rate in late stage iguana embryos (group 5-6,  $N=5$ ). Hypoxia caused a tachycardia that was unaffected by injection of atropine (Atr).

**Figure 7B** Injection of propranolol (Prop) caused an overall reduction in heart rate and uncovered a hypoxic bradycardia (group 5-6,  $N=4$ ).

**Figure 8.** Mean values ( $\pm$  SEM) of heart rate for hatchling iguanas subjected to hypoxia (10%  $O_2$ ) and hypercapnia (5%  $CO_2$ ), before (A,  $N=7$ ) and after injections of the antagonists atropine (B,  $N=12$ ) and propranolol (C,  $N=6$ ). A) Hypoxic exposure elicited a tachycardia whereas hypercapnia exposure did not change  $f_H$ . B) Injection of atropine did not block the hypoxic tachycardia. C) Propranolol abolished the tachycardic response during hypoxia exposure.

**Figure 9.** Time course of the major changes in control of the heart of the green iguana throughout incubation and after hatching expressed as the percentage of incubation time based on days after oviposition, from laying of the eggs (0%) to hatching (100%). There is evidence of the presence of muscarinic cholinergic receptors and of adrenergic tone on the heart from an early stage of development, but an inhibitory cholinergic tone is delayed until immediately prior to hatching. Hypoxia causes a bradycardia in early embryos that is unaffected by injection of atropine. In late embryos and hatchlings it causes a tachycardia that is abolished by propranolol.

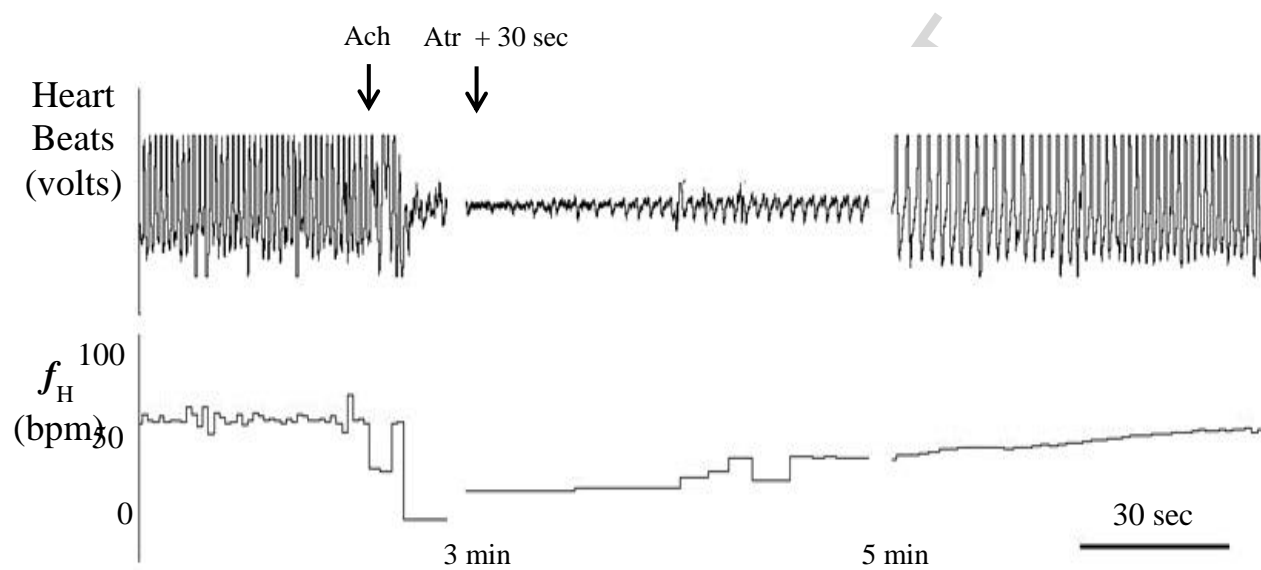
**Figure 1**

Figure 2

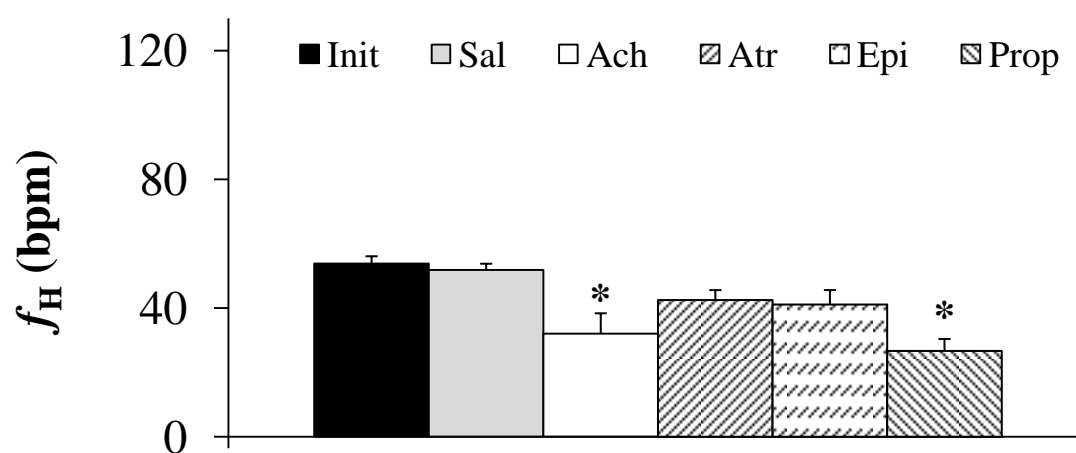
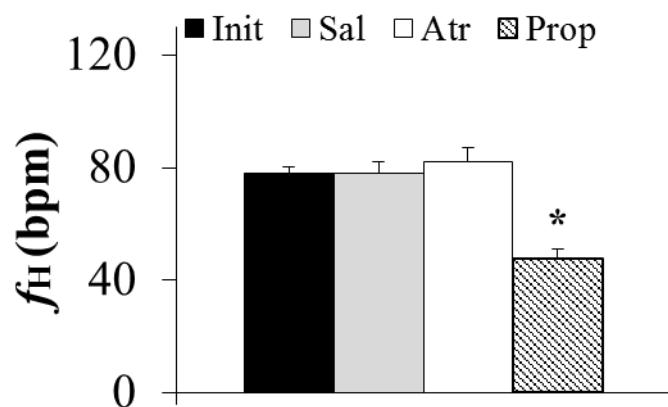
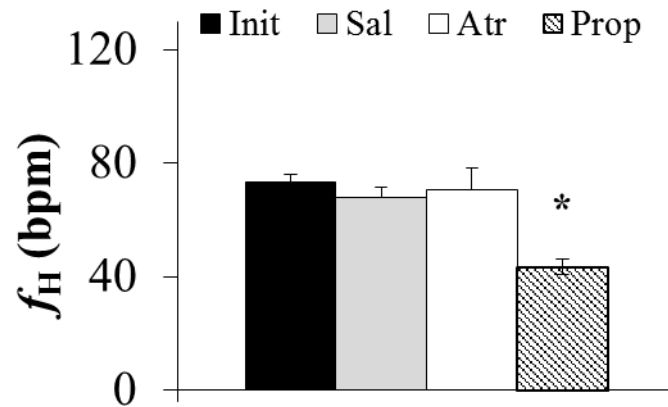


Figure 3

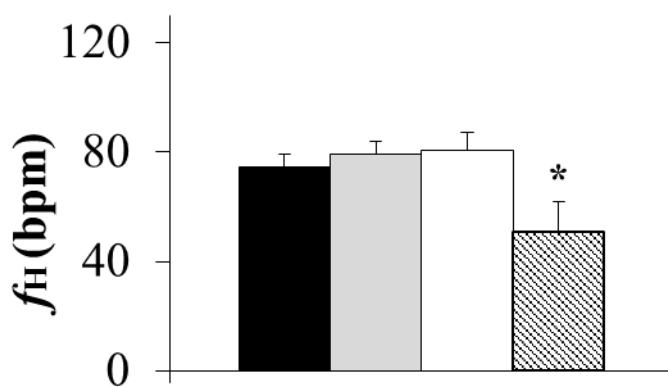
## A) Group 2



## B) Group 3



## C) Group 4



## D) Group 5

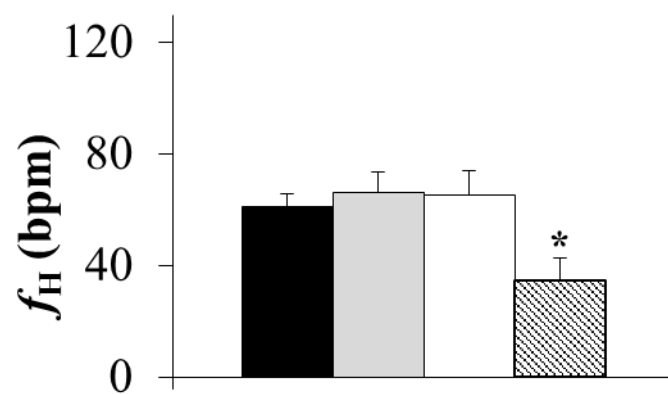


Figure 4

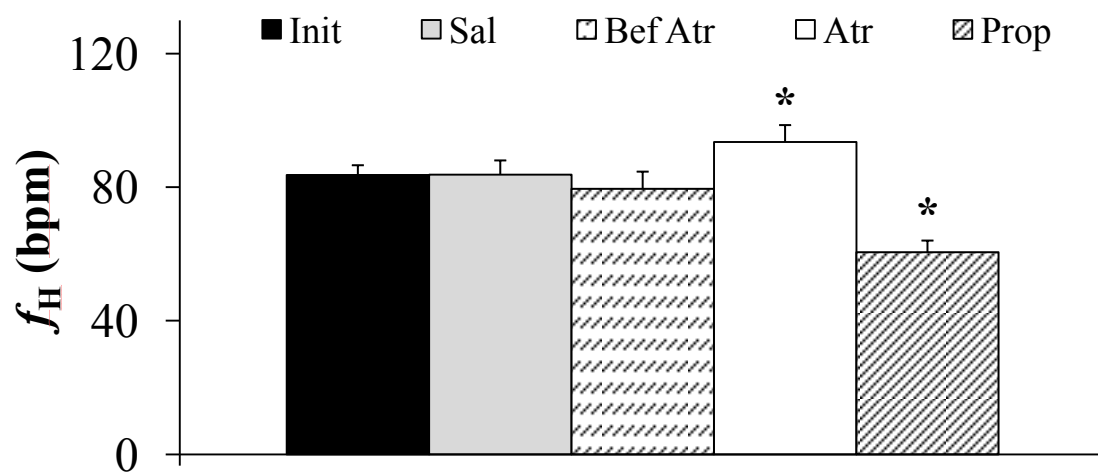


Figure 5

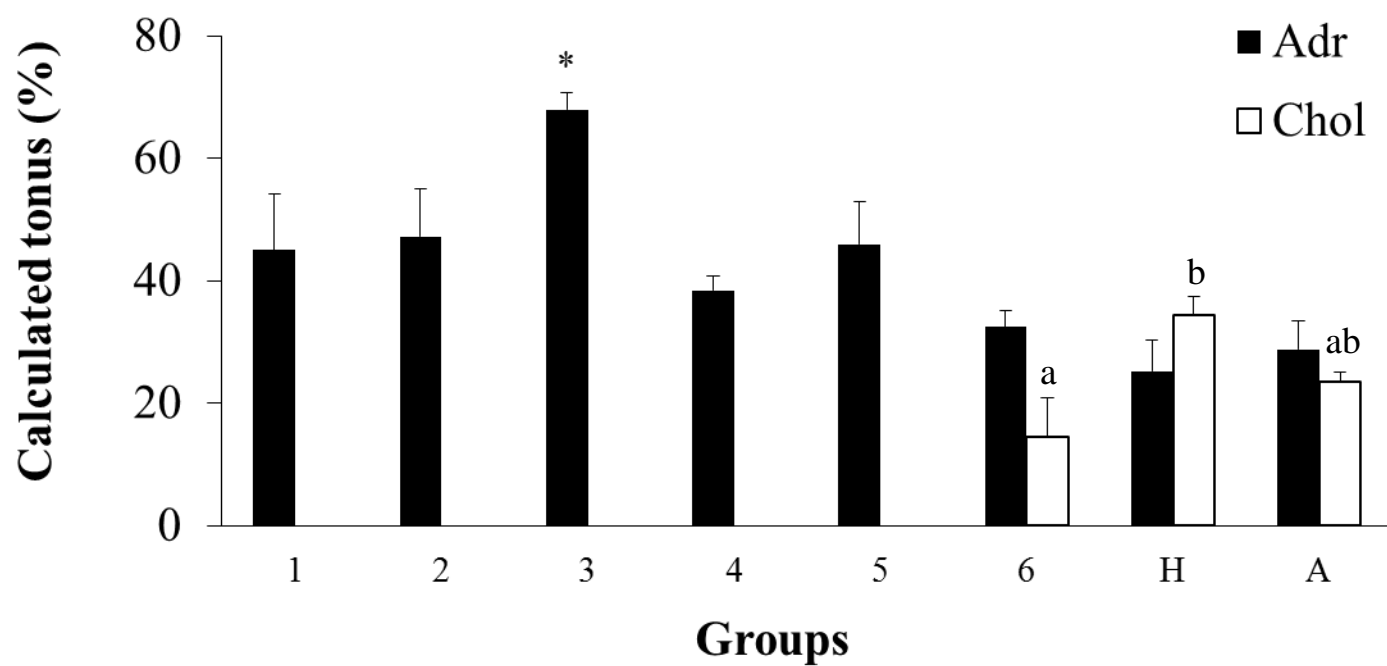


Figure 6

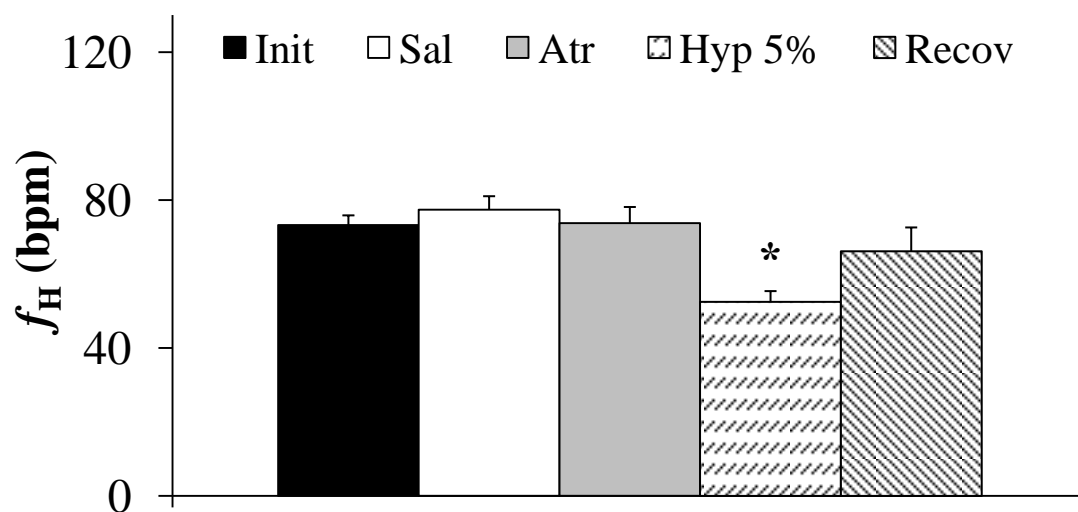


Figure 7

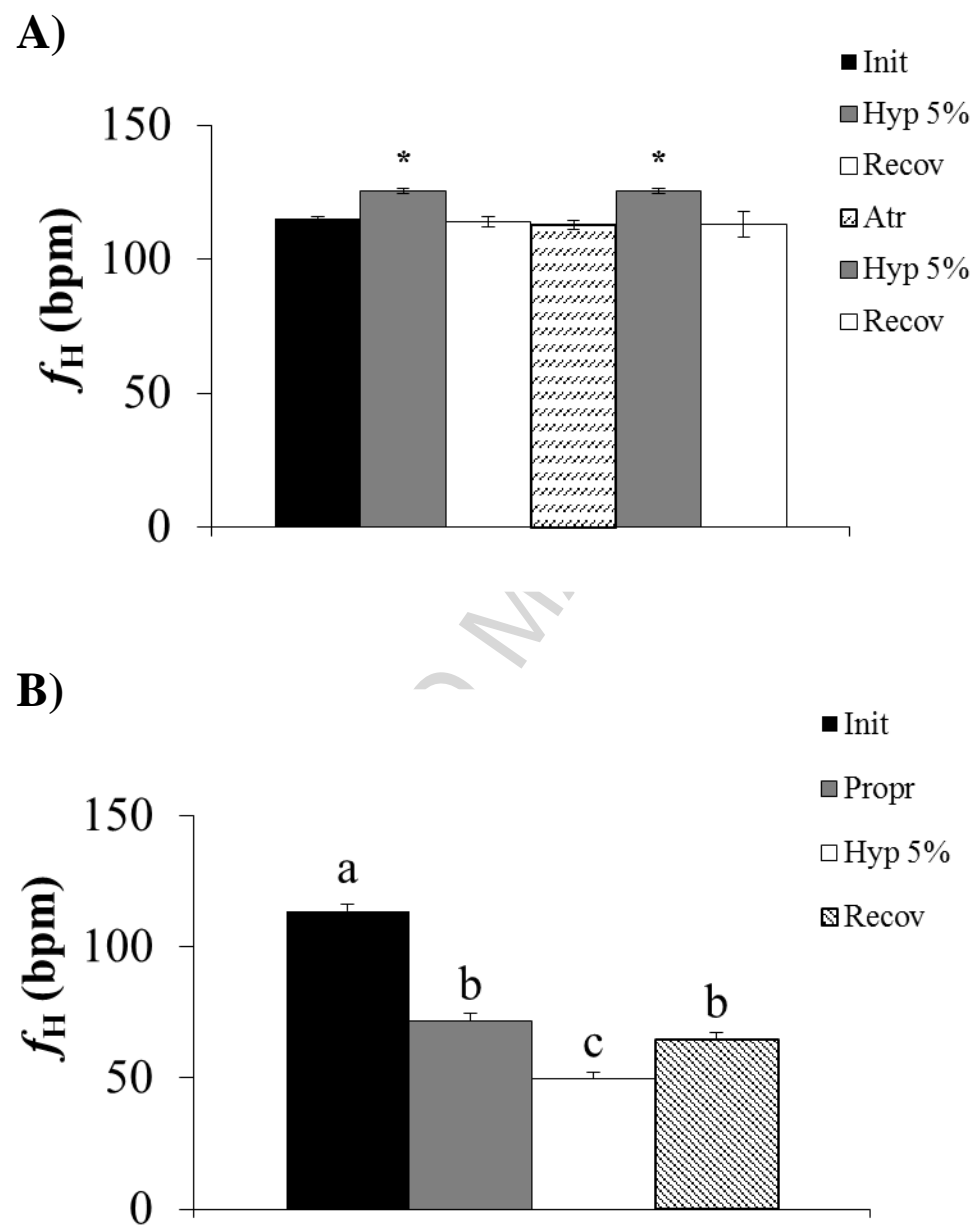




Figure 8

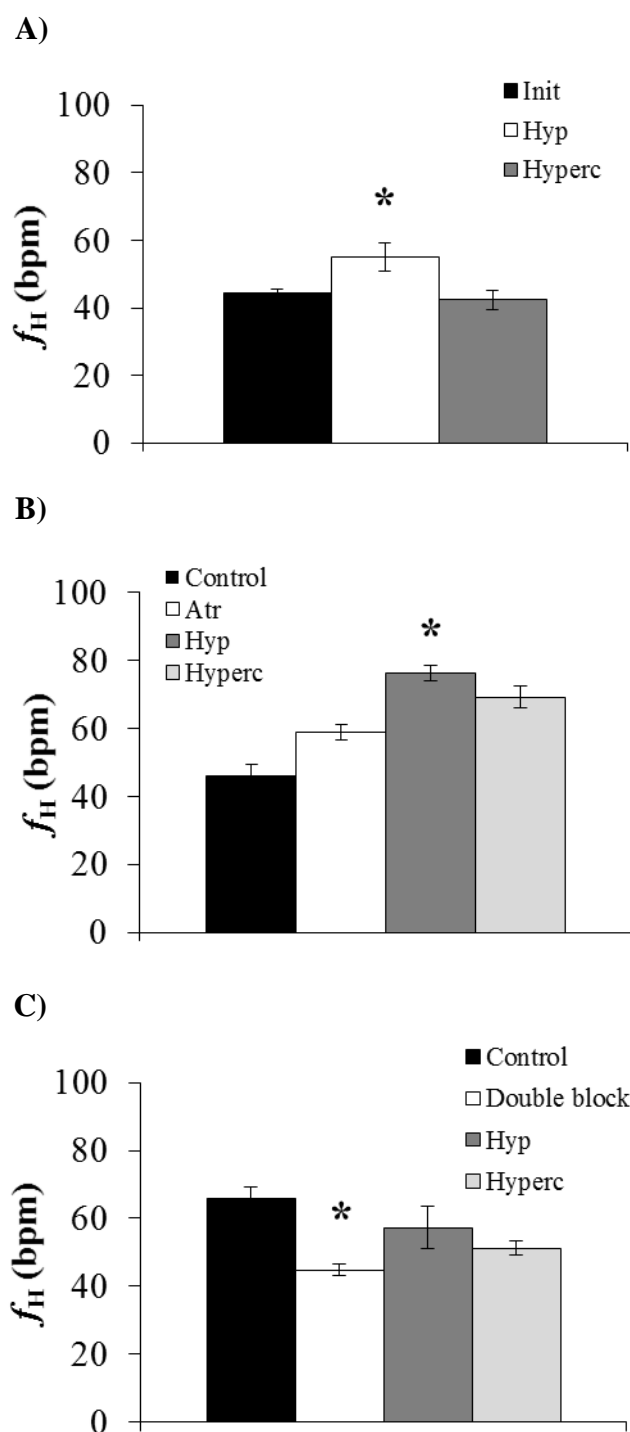


Figure 9

